Biliary cells to the rescue of Prometheus

Catherine M. Verfaillie, MD

Director Stem Cell Institute, KU Leuven
Stem Cell and Developmental Biology, Department of Development and Regeneration
Group of Biomedical Science
KU Leuven
O&N4, room 05.245
UZ Gasthuisberg
Herestraat 49
3000 Leuven
Belgium
Tel: +32 16 330295
Fax: +32 16 330294

There are no relevant conflicts of interest

Already in Greek mythology (8th-century BC), it was known that the liver has an enormous regenerative capacity, as Prometheus, punished by Zeus, was chained to a rock in the Caucasus, where an eagle was sent each day to feed on his liver that would grow back to be eaten again the next day. Thus, in contrast to many tissues, mature hepatocytes are capable of proliferating to replace damaged hepatocytes. However, when hepatocytes are no longer able to proliferate, "putative" progenitor cells can generate mature hepatocytes. The nature of these cells remains however a matter of debate (reviewed in [1]). In rodents "oval cells" thought to originate from proliferating cells in the canals of Hering upon severe injury of the liver, were identified as possible hepatocyte progenitor cells[2]. Similar observations have been made in humans, where these cells have been named "ductular reactive cells"[3, 4]. Although this was confirmed by some recent elegant CRE-LOX lineage tracing studies in mice[5, 6], other recent studies have disagreed with this concept, demonstrating that progenitor cells only minimally generate hepatocytes[7], or not at all[8, 9]. However, liver damage incurred in these murine models is usually not more than 65%-70%. That the degree with which cell loss is occurring may affect mechanisms of regeneration was demonstrated in the murine endocrine pancreas, where following a 70%-80% loss of β-cells, β-cell proliferation was the chief mechanism of β-cell replacement[10]. However, when nearly all β-cells were deleted, interestingly, α-cells transdifferentiated to different degrees to replace the β-cell compartment [11].

In two studies published recently in Gastroenterology, Jianbo He *et al.,* [12] and Tae-Young Choi *et al., (Ref)* demonstrate that nearly all biliary cells in *Danio Rero* can transdifferentiate into hepatocytes. This occurs only when nearly all hepatocytes have been eliminated (extreme deletion) by administration of metronidazole (MTZ) to transgenic fish larvae as well as adult fish, wherein the bacterial nitroreductase (NTR) is incorporated downstream of either the mature hepatocyte liver-type fatty acid binding protein (lfabp) or fatty acid binding protein-10a promoters *[Tg(lfabp:mCherry-NTR), Tg(fabp10a:NLS-mCherry),* respectively]. By contrast, under normal homeostatic conditions, biliary cells do not contribute to the parenchymal liver fraction. However, differences were seen between the two studies in the contribution of biliary cells to hepatocyte regeneration with severe but not extreme levels of tissue damage, where biliary cells contributed in the Choi *et al.,* study, but not the He *et al.,* study.

Both studies demonstrate that not solely small immature biliary cells (oval cell/ductular reactive cells in mammals), but most, if not all biliary cells, contribute to hepatocyte regeneration in this model, as they found co-staining of mCherry labeled hepatic cells with the biliary cell marker, ALCAM expressed on all biliary cells. In addition, both groups used genetic means to prove conversion of mature biliary cells into hepatocytes, by crossing *lfap-NTR* or *fabp10a-NTR* fish with transgenic fish wherein biliary cells were marked by the *Tp1* element (whose regulatory sequences directs expression of an inducible Cre recombinase following Notch activation[13]) and a third fish wherein a fluorochrome (*dsRed2* or *GFP*) was flanked by *LoxP* sites. Using such triple transgenic fish *(Tg(fabp10a:CFP-NTR; ubi:loxP-GFP-loxP-mCherry; Tp1:CreERT2)* Choi *et al.,* demonstrated that most hepatocytes in severely ablated larvae or 36h after MTZ administration were GFP positive, demonstrating that they originated from Tp1 expressing biliary cells. Likewise He *et al.,* using *Tg(lfabp:DenNTR; Tp1:CreERT2; lfabp:loxP-STOP-loxP-DsRed2)* demonstrated that most if not all hepatocytes at 36h after severe ablation of hepatocytes were DsRed2 positive[12]. Both studies demonstrate that similarly extreme hepatocyte ablation in mature fish also results in nearly complete biliary cell derived hepatocyte regeneration. A significant delay in hepatocyte regeneration was seen in fish that were treated with very low doses of the cholangiodestructive toxicant methylenedianiline (DAPM) even if no detectable signs of

biliary cell damage, demonstrating the requirement of healthy biliary cells for hepatocyte regeneration.

Partially consistent with the murine study wherein *Sox9* expressing progenitor cells were shown to play an important role in hepatocyte regeneration following extensive liver damage, He *et al.,*[12] demonstrate here using immunostaining and genetic means (sox9b^{fh313} mutants[14]) that regeneration of hepatocytes is derived from *Sox9b* expressing cells, even if this requires that mature Alcam1/Tp1 positive, *Sox9b* negative biliary cells de-differentiate to cells expressing *Sox9b*, strongly suggesting that an initial step of differentiation to bipotential progenitor cells is required prior to differentiation to hepatocytes. Consistent with this notion is the observation that additional TFs not present in mature biliary cells become re-expressed when biliary cells re-acquire a progenitor phenotype before differentiation to hepatocytes (*Hnf4a, hHex, Foxa3*).

Some of the mechanisms underlying this de-differentiation, re-differentiation and subsequent hepatocyte expansion were also addressed. When the γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester) was added before liver damage was induced with MTZ, significantly decreased hepatocyte regeneration was seen and significantly lower levels of *Sox9b* expression were detected in biliary cells. This was confirmed using *cq5* mutant fish wherein bile ducts are nearly absent[12], where hepatocyte regeneration did not occur following MTZ treatment. Thus, dedifferentiation of biliary cells to progenitor cells appears to depend on Notch signaling. These results are consistent with what has been seen in higher mammals. Notch is known as a default inducer of biliary specification, as for instance the paucity of bile ducts seen in patients with a mutation in the Notch ligand, Jagged-1 (Allagille syndrome) is caused by impaired biliary differentiation. In addition, in mice defective in Notch receptors or RBP-Jκ, liver damage is associated with poor generation of a ductular response [15].

By contrast, *Wnt2bb* deficiency did not affect de-differentiation nor re-differentiation, but inhibited the subsequent proliferation of hepatocytes. This is partly consistent with the known phenotype of *Wnt2bb* deficient fish, which exhibit delayed liver growth. However, effects of *Wnt2bb* during development appear to be more related to specification of endoderm to hepatocytes[16, 17]. The fact that Wnt2bb enhanced hepatocyte regeneration in fish is consistent with the observation that Wnt/β-catenin signaling is required for oval cell and subsequent hepatocyte generation[18, 19], even if in these models relatively modest loss of hepatocytes appeared sufficient for β-catenin-dependent oval cell activation, whereas in the zebrafish model, *Wnt2bb* only became activated following very severe hepatocyte loss.

What do these studies teach us regarding hepatocyte regeneration following liver damage?

The studies point to the significant influence the type or extent of defect caused to a tissue has on repair mechanisms. This is also consistent with what has also been shown for other tissues such as endocrine β-cells[10, 11].

Second, the studies demonstrate, at least in the model-organism *Danio Rero,* that following extreme hepatocyte loss, complete replacement of hepatocytes occurs by a process of de-differentiation and then re-differentiation of mature biliary cells throughout the liver, and this by re-using signal mechanisms known to also play a role in the development of the biliary and hepatocyte compartment. Of note, among the TFs that became re-expressed in biliary cells as part of the dedifferentiation process are Hnf4α and Foxa3, both being reported to be capable in combination with other

transcription factors, to reprogram mouse fibroblasts to hepatocytes[20]. Therefore further exploration of the phenotype of the "dedifferentiated" biliary cells during hepatocyte regeneration may identify other important transcription factors and signaling mechanisms involved in de/transdifferentiation of cells to hepatoblasts or hepatocytes, that could be exploited for the generation of hepatoblasts and hepatocytes.

Third, similar observations have been made in some murine and human studies, even if hepatocyte loss in mice/men was significantly less than what can be achieved in *Danio Rero*[5, 6]. As discussed above, contribution of biliary-derived cells to hepatocytes when the liver was severely but not extremely damaged differed in the two studies, but mature hepatocyte proliferation persisted when hepatocyte loss was not extreme, which is not usually seen in mice. This may point to significant differences that exist in the regeneration ability of most tissues between zebrafish and mammals, endowing zebrafish hepatocytes with significant greater proliferation ability compared with mammalian cells.

Finally, compared with mouse and man, regeneration of hepatocytes from biliary cells appears also to be much more extensive in zebrafish. Whether this is due to the difference in proliferation ability of biliary cell-derived hepatocytes (as shown in the *Wnt2bb* mutant fish where hepatocyte proliferation is decreased and regeneration also decreased), or because of differences in the location and number of bile ducts (and their precursors) remains to be determined. However, like insights in signals that induce de-differentiation may aid in development strategies to create hepatocytes/hepatoblasts of non-liver derived cells may aid in generation of hepatocytes for pharmaceutical and regenerative purposes, further insights in how hepatoblasts generated by the differentiation process can be fated towards proliferating hepatocytes will likely yield novel approaches to treat acute as well as chronic liver diseases.

REFERENCES

1. Michalopoulos GK. Liver regeneration: alternative epithelial pathways. Int J Biochem Cell Biol 2011;43:173-9.
2. Petersen BE, Zajac VF, Michalopoulos GK. Hepatic oval cell activation in response to injury following chemically induced periportal or pericentral damage in rats. Hepatology 1998;27:1030-8.
3. Roskams TA, Theise ND, Balabaud C, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology 2004;39:1739-45.
4. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. Mech Dev 2003;120:117-30.
5. Furuyama K, Kawaguchi Y, Akiyama H, et al. Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 2011;43:34-41.
6. Carpentier R, Suner RE, van Hul N, et al. Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells. Gastroenterology 2011;141:1432-8, 1438 e1-4.
7. Huch M, Dorrell C, Boj SF, et al. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. Nature 2013;494:247-50.
8. Espanol-Suner R, Carpentier R, Van Hul N, et al. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 2012;143:1564-1575 e7.
9. Malato Y, Naqvi S, Schurmann N, et al. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J Clin Invest 2011;121:4850-60.
10. Dor Y, Brown J, Martinez OI, et al. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41-6.

11. Thorel F, Nepote V, Avril I, et al. Conversion of adult pancreatic alpha-cells to beta-cells after extreme beta-cell loss. Nature 2010;464:1149-54.
12. He J, Lu H, Zou Q, et al. Regeneration of Liver after Extreme Hepatocyte Loss Occurs Mainly via Biliary Transdifferentiation in Zebrafish. Gastroenterology 2013.
13. Parsons MJ, Pisharath H, Yusuff S, et al. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. Mech Dev 2009;126:898-912.
14. Delous M, Yin C, Shin D, et al. Sox9b is a key regulator of pancreaticobiliary ductal system development. PLoS Genet 2012;8:e1002754.
15. Fiorotto R, Raizner A, Morell CM, et al. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. J Hepatol 2013;59:124-30.
16. Lancman JJ, Zvenigorodsky N, Gates KP, et al. Specification of hepatopancreas progenitors in zebrafish by hnf1ba and wnt2bb. Development 2013;140:2669-79.
17. Ober EA, Verkade H, Field HA, et al. Mesodermal Wnt2b signalling positively regulates liver specification. Nature 2006;442:688-91.
18. Sekine S, Gutierrez PJ, Lan BY, et al. Liver-specific loss of beta-catenin results in delayed hepatocyte proliferation after partial hepatectomy. Hepatology 2007;45:361-8.
19. Tan X, Behari J, Cieply B, et al. Conditional deletion of beta-catenin reveals its role in liver growth and regeneration. Gastroenterology 2006;131:1561-72.
20. Sekiya S, Suzuki A. Direct conversion of mouse fibroblasts to hepatocyte-like cells by defined factors. Nature 2011;475:390-3.

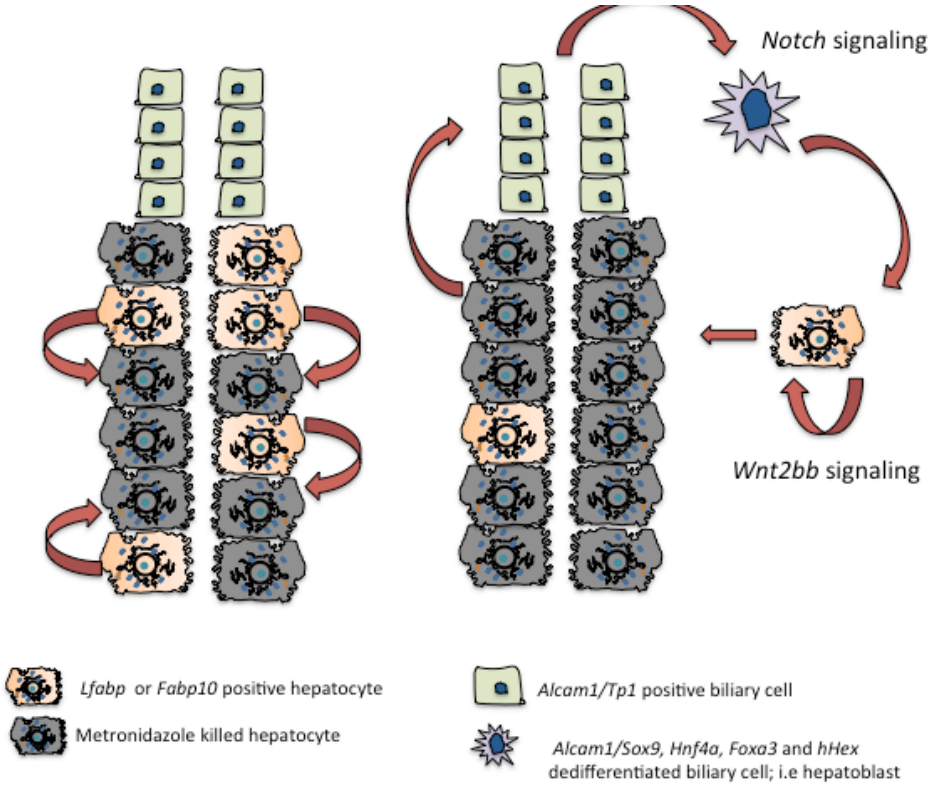

Figure legend:
Left: During severe (±60-70%) hepatocyte damage in zebrafish larvae or adult fish, Lfabp or Fabp10 positive hepatocytes multiply to generate new hepatocytes.
Right: during extreme (>95%) hepatocyte damage, nearly all mature Alcam1/Tp1+ biliary cells de-differentiate to Sox9, Hnf4a, Foxa3 and hHex - Alcam1 double positive cells, after which the to Sox9, Hnf4a, Foxa3 and hHex – Alcam1 double positive cells (hepatoblasts) after which they differentiate to a pool of still expanding mature (albumin+, glycogen storing, bile excreting) hepatocytes to replace all damaged hepatocytes in 3-5 days. Creation of hepatoblasts is Notch dependent, while expansion of newly generated hepatocytes is *Wnt2bb* dependent.